Interaction of coagulation factors

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Chapter 3

Interaction of Coagulation Factors

H.C. HEMKER

Medical Faculty Maastricht, Department of Biochemistry, Biomedical Centre, Beeldsnijdersdreef 101, Maastricht (The Netherlands)

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Chapter 3

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H.C. HEMKER

Medical Faculty Maastricht, Department of Biochemistry, Biomedical Centre, Beeldsnijdersdreef 101, Maastricht (The Netherlands)

1. INTRODUCTION

The interaction of coagulation factors is an exciting yet poorly studied field in enzymology. Its charm lies in the unique ensemble of enzymatic actions comprised in the reaction mechanism of blood coagulation. On the one hand, it shows beautiful examples of well-documented modes of interaction of proteins such as limited proteolysis. On the other, it appears that in blood coagulation some ways of protein interaction occur that are hardly known in other fields of biochemistry. Coagulation shows features that can only be explained when essentially novel mechanisms of protein—protein interaction and of protein—lipid interaction are recognised.

Still, a description of the coagulation process as the sum of its dissected parts hardly does justice to the beauty and intricate refinement of the whole. The constitutive reactions are qualitatively so intertwined and quantitatively so economically patterned and well-balanced that the whole is, as a result, a unique non-linear kinetic system. This system joins the seemingly incompatible properties of immediate explosive kinetics on the one hand, and self-limiting properties on the other. In this way coagulation meets the very strict demands of no leakage from wounded vessels and no thrombosis in intact vessels. Failure to meet these demands is a major drawback in the struggle for survival. Clearly the physiology of vessels and platelets is as important here as coagulation. Yet it must be recognised that thrombin plays a key role in these fields [49,60,63]. Therefore the study of the coagulation mechanism is by no means just the study of the generation of a fibrin clot.

Insight into the mechanism of thrombin generation is essential for an understanding of the processes of haemostasis and thrombosis. The antihaemophilic factors play a central role in thrombinogenesis.

It must be borne in mind that it is not only at the level of thrombin that coagulation interacts with other important physiological systems. Factor-XII (Hageman factor) activation not only triggers off intrinsic coagulation, but

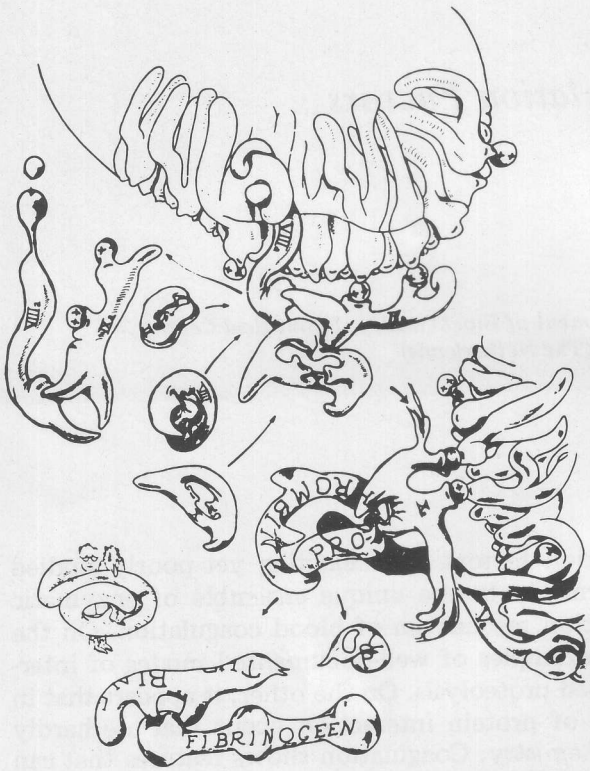

Fig. 1. An artist's view on the interaction of blood coagulation factors. After an original drawing by Leo Vroman.

appears to play a role in the kinin system, the complement system and in fibrinolysis as well [1,40,52,54,65].

Given the medical importance of haemorrhage and thrombosis — more than half of all deaths being caused or complicated by either — the study of the interaction of blood coagulation factors imposes itself as a medical necessity. But even if it did not, the study would still be fully justified on biochemical grounds. Conditions in coagulating plasma differ essentially from those prevailing in enzymatic systems studied in the laboratory. This necessitates a reappraisal of the basic notions employed in enzymology: a wholesome exercise for the biochemist who gets entangled in this field [26].

2. DEFINITIONS AND BASIC NOTIONS

To avoid confusion it seems advisable to begin with a definition of some seemingly well-known terms [7,12].

An *enzyme* is a protein with catalytic properties capable of converting some molecule (in coagulation always another protein) by changing covalent

bonds. In coagulation enzymes always break bonds: the coagulation factors with enzymatic properties are proteolytic enzymes. Factor $XIII_a$ off the main coagulation pathway is the only exception.

Enzymes convert a *substrate* into a *product*. In coagulation the substrate is often a proenzyme and the product itself is then an enzyme. It is therefore confusing to speak of any factor as a substrate or as an enzyme unless the reaction in which it plays such a role is clearly defined. Most coagulation factors are substrates in one reaction and enzymes in another.

In coagulation the term 'substrate' is used in still another way. It is sometimes said that, say, the factor-VIII activity of some preparation is tested using a factor-VIII deficient *substrate*. This means that the factor-VIII deficient plasma is used as a *reagent* for factor-VIII activity, i.e., to constitute a reaction mixture in which clotting time depends upon the concentration of factor VIII.

These definitions of enzyme and substrate are current in general enzymology. This does not automatically imply that the kinetics of substrate conversion will be those known from the textbooks. The kinetics developed in general enzymology presuppose conditions not necessarily prevailing in blood coagulation experiments. Examples are the assumption of excess of substrate over enzyme and the measuring of initial reaction velocities. Some notions from regular kinetics remain useful.

The *Michaelis constant* (K_m) is defined as the concentration of a reactant at which half the maximal reaction velocity is obtained. *Maximal reaction velocity* (V_{max}) is the reaction velocity that would be obtained at an infinite concentration of the varying reactants in a given reaction mixture (i.e., at a fixed concentration of the other reactants). In coagulation, the reactant for which the K_m can be defined may be the enzyme as well as the substrate [26].

The conversion of a proenzyme into an enzyme will be called *activation*. When the proenzyme is a coagulation factor (indicated by a roman numeral) the activated factor will be indicated by the subscript *a* (e.g. thrombin = factor II_a).

Some factors (e.g. factors V and VIII) can have their activity enhanced

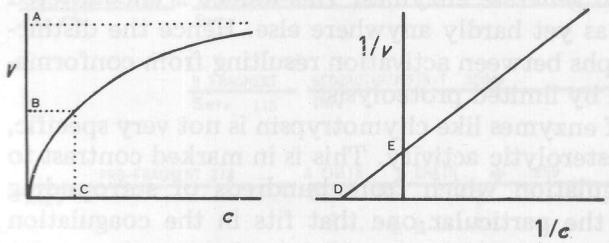

Fig. 2. Basic graphs in enzyme kinetics. The concentration (c) in coagulation can be substrate or enzyme concentration. Meaning of the intercepts A, V_{max}; B, ½ V_{max}; C, K_m; D, $-1/K_m$; E, $1/V_{max}$.

without obtaining enzymatic properties. This will be called enhancement and indicated by the subscript *e*. Finally the inactivated factors will be given the subscript *i*.

3. CLASSIFICATION OF INTERACTIONS

The ground for all enzymatic activity is that the stereochemical structure of amino acid side-chains of the enzyme protein at one circumscribed place in the molecule allows the substrate to bind and undergo changes that are very unlikely in free solution. The site where the substrate binds and is converted into product is called the *active site* of the enzyme.

The structure of the active site is determined not only by the presence of specific side-chains but also by the particular juxtaposition of these residues. This juxtaposition is in turn determined by the whole of the tertiary structure of the enzyme and will change with conformational changes on the part of the enzyme. The generation of enzymatic activity in a previously inactive protein molecule occurs because a conformational change in the protein causes some side-chains to move and to attain the structure that forms the active site (for a review see ref. 32).

Such changes are best documented in proteins from the chymotrypsinogen group. Chymotrypsinogen is a protein containing the essential residues for forming an active proteolytic centre in an unfavourable configuration. When a specific covalent bond in this molecule is split, the previously stable tertiary structure becomes unstable and changes to a new one. Now some defined residues do constitute an active centre capable of proteolytic and esterolytic activity. Among the active residues the major role is played by a seryl group (hence the name 'serine esterase' for this group of enzymes) in close cooperation with two histidyls. It is important to realise that in the conversion from zymogen into enzyme there exists a transient state of the molecule in which the covalent bonds are already broken while the enzymatically active tertiary structure is not yet attained: it is thus essentially a change in tertiary structure that causes enzymatic activity. It is therefore possible that changes in tertiary structure alone, not involving the breaking of covalent bonds are able to generate enzymes. This indeed is encountered in blood coagulation — and as yet hardly anywhere else. Hence the distinction in the following paragraphs between activation resulting from conformational changes and activation by limited proteolysis.

The proteolytic activity of enzymes like chymotrypsin is not very specific, and the same is true of its esterolytic activity. This is in marked contrast to the enzymes in blood coagulation which from hundreds of surrounding molecules often pick only the particular one that fits in the coagulation pathway. This can hardly be achieved by specificity of the active centre alone. Discrimination on the part of the active centre is limited to structures of the order of magnitude of the active centre, say of 15 Å diameter. The

specificity required in blood coagulation concerns protein molecules and thus requires binding sites of the order of magnitude of 50—150 Å; accessory binding groups are therefore necessary. The latter are in part located on proteins other than the enzyme itself. Proteins adsorbed next to the enzyme at a phospholipid—water interphase provide these sites. We call them *paraenzymes* [24]. Together with the protein carrying the active site they form complex enzymes. Hence paragraph 5.

The paraenzymes undergo changes in activity induced by thrombin discussed in paragraph 6.

Inhibitory reactions — important though rather neglected until now — are the subject of paragraph 7 and a preliminary glimpse of the mechanism functioning as a whole is offered in the last paragraph.

4. ACTIVATION OF PROENZYMES

(a) Limited proteolysis

The best documented example in blood coagulation of activation by limited proteolysis is the conversion of prothrombin into thrombin [20,35,45,47,57]. It will be briefly summarized here because it probably is a good model for other activations occurring in coagulation, notably for that of factor IX.

Prothrombin is a single-chain protein with a molecular weight of 73 000. This chain has four parts tentatively called A-Fragment, B-Fragment, Thrombin-A Chain and Thrombin-B Chain. We adopt the nomenclature of Magnusson here [45].

During activation the amino acid chain is cleft and the parts are separated. The A- and B-Fragments remain attached to each other by means of a disulphide bridge. Each of the four parts has a specific function. The A-Fragment contains the Ca-binding sites that via Ca^{2+} fix prothrombin to a

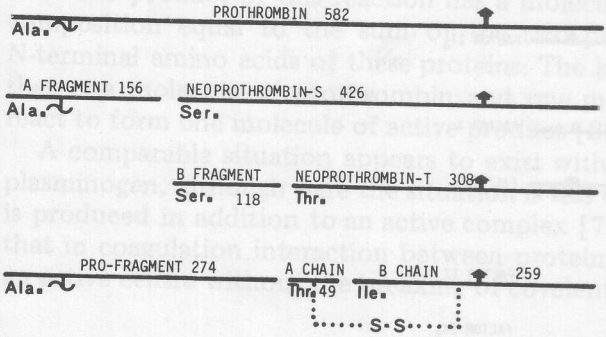

Fig. 3. The breakdown of prothrombin (after Magnusson, ref. 45). The numbers refer to amino acid residues. The arrow indicates the active serine, the squiggle the Ca^{2+} binding site.

phospholipid—water interphase [14,36,58,74]. This is an essential feature of the activation mechanism, as we shall see below.

The B-Fragment shows an affinity to factor V [34]. The Thrombin-A Chain shows no homologies to known esterases but probably plays a role in determining the specificity of thrombin. The Thrombin-B Chain contains the active serine and histidines and also in other respects shows extensive homologies with chymotrypsin, trypsin, elastase and other known serine esterases [19]. The breaking of the bonds between the Thrombin-A Chain and the Thrombin-B Chain induces in the Thrombin-B Chain the conformational changes that lead to the formation of an active centre. The Ca-binding sites in the A-Fragment are γ-glutamyl carboxylic acids [75]. They are not built into the protein during ribosomal synthesis [71,72]. The product of ribosomal synthesis is a precursor protein with pairs of ordinary glutamic acid residues in the A-Fragment part. By a postribosomal vitamin K-dependent step glutamic acid residues are modified into γ-glutamyl carboxylic acid residues. In the absence of vitamin K or during administration of Vitamin K antagonists the precursor enters the blood where it can be recognized as the so-called PIVKA-II (Protein Induced by Vitamin K Absence analogous to Factor II) [31].

There are extensive homologies between the coagulation factors II, VII, IX, and X [15,16,45]. All four of them are synthesized as precursors that need finalization in a vitamin K-dependent step. All four are serine esterases but with distinctly different active sites, as can be inferred from their different susceptibilities to different serine esterase inhibitors [43].

Factor II differs from the other three in that in its active form it does not have the Ca^{2+} binding groups of the A-Fragment. These groups are essential for the functioning of factors VII_a, IX_a, and X_a [36,58]. After activation of these factors the part homologous to the A-Fragment thus remains attached to the part comparable to the Thrombin-B Chain.

This is illustrated in Fig. 4. This figure should not be taken to indicate

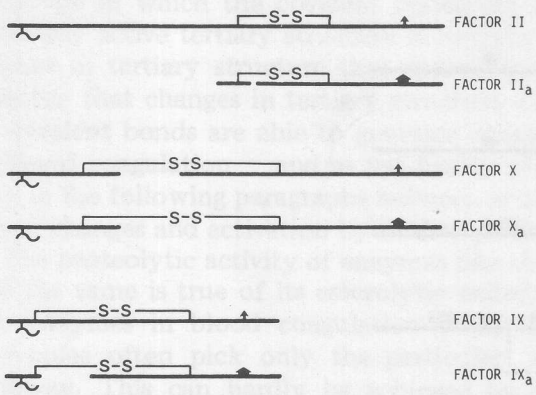

Fig. 4. Analogies between clotting proteases (after Magnusson, ref. 45). The arrows indicate the active serine (bold: activated; small: not activated). The squiggle is the Ca^{2+} binding site.

that one proenzyme can give rise to either thrombin or to factor VII_a, IX_a, or X_a. This suggestion, which may be found in the older literature [69], is no longer tenable as each proenzyme (factor) together with its precursor (PIVKA) has clear-cut immunological identity and because, as stated above, the enzymes show distinct differences in their active sites [66].

Of particular interest in the present context is the activation of factor IX. Data on the details of the reaction in this particular factor are scarce. As yet there are no reasons to assume that it differs considerably from the general scheme outlined above [15,16]. The activating enzyme in the case of factor IX is the product of the interaction of factors XII and XI. It has been claimed that both factors engage in proteolytic and esterolytic activity. Detailed information as to the nature of the factor IX-activating enzyme is conflicting [8,17,39,41,44,55].

(b) Activation without proteolysis

As outlined above, the essential feature of the conversion of a proenzyme into an enzyme is the change of tertiary structure bringing about the correct juxtaposition of the amino acid residues in the active centre. Splitting-off a part from the amino acid chain is not the only way in which the tertiary structure may be influenced. When a protein adsorbs onto an interphase its tertiary structure changes. In factor XII this results in the formation of an active centre.

The desorbed protein seems to retain its activity for some time, such retention being due to complex formation of factor XII_a with factor XI [17,73]. Another school claims however that factor XI activated by factor XII_a is itself a serine esterase [41]. The exact nature of the active product (contact product: C.P.) that results from the interaction of factors XII and XI and a wettable surface does not appear to be determined.

A very interesting feature of coagulation is the activation resulting from complex formation between two proteins. This has been most clearly shown with reference to the interaction between prothrombin and staphylocoagulase. The product of this reaction has a molecular weight and an amino acid composition equal to the sum of the two proteins involved and has the N-terminal amino acids of these proteins. The kinetics of its formation show that one molecule of prothrombin and one molecule of staphylocoagulase react to form one molecule of active product [22].

A comparable situation appears to exist with respect to streptokinase and plasminogen, although here the situation is less clear because normal plasmin is produced in addition to an active complex [77]. But it is clear in any case that in coagulation interaction between proteins can cause the formation of an active centre without the breaking of covalent bonds.

5. COMPLEX ENZYMES AT INTERPHASES

Phospholipid micelles participate in the coagulation reaction sequence by

offering a phospholipid—water interphase to the interacting coagulation factors.

Again the best documented interactions are to be found in the final steps of the coagulation pathway, i.e., the interaction of the factors V_e, X_a, and II [2,9,14,18,23,28,30,58]. It has been shown that these three proteins all bind to the interphase. Both factor II and factor X_a possess γ-carboxyglutamyl residues that via Ca^{2+} presumably bind to anionic sites at the interphase. This may partly explain the correlation between the charge of the micelles and their thromboplastic activity. Model studies of factor V with hydrophobic powders make it probable that factor V binds to phospholipid interphases by hydrophobic interaction [38]. The fact that micelles from homogeneous phospholipids are much less active in coagulation than mixed ones may be due to the generation of favourable mosaic patterns of hydrophilic and hydrophobic patches in the latter case [24]. The kinetics of the formation of prothrombinase support the hypothesis that one molecule of factor X_a absorbed next to one molecule of factor V forms one molecular unit fit for prothrombin conversion [23]. Prothrombin will fit into this complex by binding to the phospholipid with its A-Fragment and to factor V with its B-Fragment. This fixation probably reduces freedom of movement in the vulnerable sites of the prothrombin relative to the active site of factor X_a, thus enhancing the efficiency of factor X_a action. Factor X_a in free solutions can also activate factor II but at a pace of less than 1% of that of the equivalent amount of prothrombinase complex [42].

The interaction between the factors $VIII_e$ and IX_a and phospholipid micelles that form the factor-X activating enzyme is again much less well known than the generation of prothrombinase. Yet evidence has been presented that the antihaemophilic factors interact in a way precisely analogous to that described for the factors X_a and V_e, and that they form a factor-X activating enzyme in this way [28,33,56]. If this is true, the factors IX_a, $VIII_e$ and X form a trimolecular complex at the interphase, from which factor X_a results.

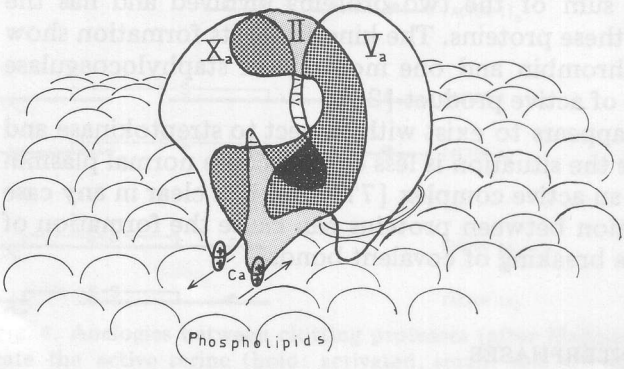

Fig. 5. Prothrombin and prothrombinase at a water—lipid interphase.

It may even be the case that factor X_a does not detach from the surface but directly complexes with factor V_e to form the prothrombinase moiety described above. This is the more likely as proteins adsorbed onto a lipid—water interphase retain a fair degree of freedom of movement in the plane of the interphase [78].

It is clear that direct measurement of proteins adsorbed onto interphases, e.g. by ellipsometry, is of prime importance for the further studying of these phenomena [50].

6. ENHANCEMENT

No enzymatic actions of factors V and VIII have been demonstrated. They appear to function only as paraenzymes to the factors X_a and IX_a. Yet some kind of activation of these factors does take place. The activity of both factors is markedly enhanced by a low concentration of thrombin [4,51,62,64]. Yet, in the strict sense no enzymatically active centre is formed in these proteins. We therefore propose the term *enhancement* for this type of activation. In symbols: V becomes V_e and VIII becomes $VIII_e$. The nature of the enhancement of factor VIII is not yet clear. More details are known about factor V that again may be of use as a model although here too much is uncertain.

It has been shown [11,38] that human factor V exists in a 480 000 mol. wt. form that can spontaneously convert into a 120 000 mol. wt. form. This has been interpreted in terms of the splitting of a tetramer which presumably is reversible. Thrombin and a fraction from Russell's Viper Venom bring about an irreversible transition into the low molecular-weight form. Given their nature one would expect such enzymes to act by limited proteolysis. Indeed small molecular-weight differences between factor V treated with thrombin and untreated factor V have been reported [11].

Such activation appears not to be essential for factor V to function in the coagulation system. It is, however, essential for the functioning of factor VIII [64]. The form of factor V with enhanced activity is much more labile than the untreated form, and the thrombin-treated form even more so than the RVV-treated one. It has been shown that the inactivation of factor V results from the reaction of two molecules of factor V_e [29]. This leads to the following scheme:

$$\text{factor V} \rightleftharpoons 2\ \text{factor V}_e \xrightarrow{\text{thrombin or RVV}} 2\ \text{factor V}_e \rightleftharpoons \text{factor V}_i$$

Although the subunit structure of factor VIII is currently a matter of considerable interest, it can be seen elsewhere in this book that the role of these subunits in the coagulation mechanism and the exact nature of the action of thrombin are still obscure.

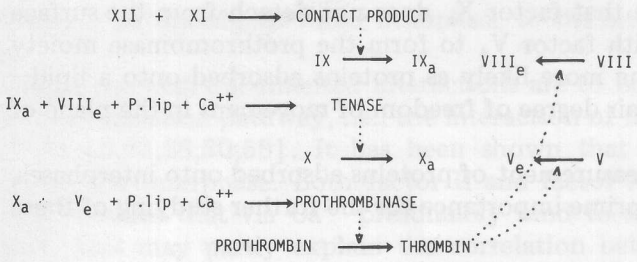

Fig. 6. A biochemist's view of the interaction of clotting factors. The roman figures indicate the corresponding coagulation factors. P.lip., phospholipid. Only the intrinsic pathway is shown.

A scheme of the various interactions that give rise to thrombin formation via the intrinsic pathway is given in Fig. 6.

7. INHIBITORY REACTIONS

The serine esterases among the coagulation factors II_a, VII_a, IX_a, and X_a all seem to interact with antithrombin 3 although for some factors (II_a and X_a) this has been shown more definitely than for others [3,10,13,79]. Factor XI_a is also reported to be inactivated by antithrombin 3 [68].

Such inhibiting reactions are extremely important features of the coagulation system. A 50% reduction of antithrombin 3 leads to a pronounced tendency to thrombosis [48], and complete absence is not encountered possibly because it is a lethal mutation.

α_2-Macroglobulin and 1-antitrypsin also inhibit active coagulation factors [67].

Other inactivation mechanisms exist as well. We already mentioned the spontaneous dimerisation of enhanced factor V to an inactive product. Thrombin thus leads to a burst of relatively short-lived activity on the part of factors V and VIII. This is one of the features that determines the non-linear character of the coagulation mechanism (see below).

Still another type of inhibiting reaction may result from the generation of breakdown products of proenzymes (i.e., factors II, VII, IX and X). An inhibitory activity has been ascribed to the A- and B-Fragments of prothrombin [46]. Whether this is a novel activity on the part of these polypeptides or simply a cause of their affinity for phospholipids and factor V is as yet unclear.

A final group of reactions inhibiting the coagulation process is the breakdown of many factors by plasmin. Apart from fibrin, at least factors II, V, and VIII are consumed by plasmin. The coagulation-inhibiting action on the part of excess phospholipid can be simply explained by the fact that an increase of the surface of the phospholipid—water interphase reduces the chance for the interacting coagulation factors to meet. Apart from that,

some types of phospholipids — such as can be isolated from bovine brain — appear to have an inhibiting action per se [5,6]. Presumably they inhibit by provoking adsorption of the coagulation factors in a steric mode that does not generate an active complex. The same type of explanation has been proposed for the inhibitory action of polyene antibiotics [61].

8. COAGULATION AS A NON-LINEAR SYSTEM

Even if complete, the above qualitative sketch of the possible interactions of coagulation factors would not represent an exhaustive description of the coagulation system. For such a description must take into account the kinetics of the system, the more so because blood coagulation belongs to the set of systems that are complicated enough to show non-linear behaviour. This means that in these systems there exists a varying relationship between the change of some parameter (say, substrate concentration) and its effect (say, reaction velocity).

In simple systems an increase in substrate concentration (or a decrease in inhibitor concentration or an increase in enzyme concentration) will always cause an increase in reaction velocity. In non-linear systems this is not the case.

Non-linear behaviour is expected on a theoretical basis and is indeed observed in biochemical systems that exceed a certain degree of complexity. For a general view of the field see ref. 27. Sel'kov has given a review of the systems that are complex enough in principle to show this type of behaviour [70]. One of them is shown in Fig. 7, where E is the enzyme that converts X into Y. E is activated by low concentrations of Y and inhibited by a high concentration of Y. Y is converted into Y′ in a second reaction. It takes little imagination to project this scheme onto a part of the coagulation mechanism as is shown also in Fig. 7.

As we know, thrombin first enhances and then destroys factor-V activity. The product of prothrombinase activity thus both activates and inactivates its enzyme. We see that even a small subsystem of coagulation already satisfies the condition for a non-linear system; and there is no doubt that the complete system will have a complicated non-linear character.

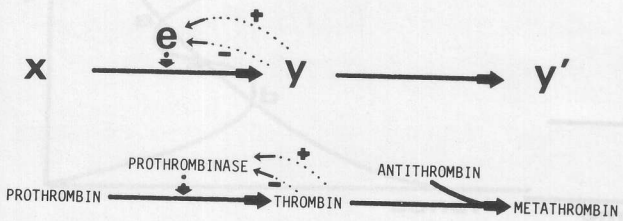

Fig. 7. A non-linear kinetic mechanism and its projection on part of the coagulation system.

On experimental grounds, too, there can be no doubt as to the non-linear character of the coagulation system. The most characteristic feature of coagulation is that the all-or-none chemical kinetics combine with a limited spatial extent of these changes. When studying coagulation in vitro one is inclined to forget that effective in vivo coagulation is limited to a confined region in space. Coagulation in vivo is a limited explosion, i.e., an explosive change that sets its own boundaries. With certain precautions this can be readily imitated in vitro [21]. The importance of the phenomenon is clear. Thrombogenesis is the result of thrombin formation beyond healthy limits and a haemorrhagic tendency is the failure to produce a sufficiently important "thrombus" at a bleeding site. I will not go into the details necessary to apply this reasoning to platelets and vessels as well. Suffice it to say that thrombin is a necessary reactant to make irreversible platelet thrombi (or, as the case may be, hemostatic plugs). Given the scope of this chapter I shall not deal with these more complex topics here, however consistent with the foregoing general scheme it would be to do so.

The explanation of the "limited explosion" must be sought in a combination of non-linear kinetics and physical transport phenomena such as bulk stream and diffusion. Again we will simplify matters by considering only diffusion as a physical mechanism for the introduction of concentration gradients.

Non-linear systems have fascinating kinetic properties including multiple steady states, excitation thresholds, spontaneous generation of dissipative structures, memories, all- or- none behaviour, etc. A combination of an excitation threshold, multiple steady states and a diffusion gradient makes it possible for a "limited explosion" to occur.

(a) Excitation threshold

Usually a system in a steady state will react to a change in the environment (perturbation) by finding a neighbouring steady state. Non-linear systems may show the same behaviour, but when a perturbation exceeds a certain threshold unexpected behaviour will result (Fig. 8).

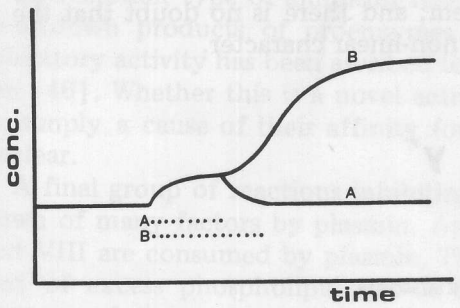

Fig. 8. Perturbation in a non-linear system. (A) below the excitation threshold. (B) above the excitation threshold.

(b) Multiple steady states

When a system shows non-linear kinetics, and the velocity of generation of the substrate by an independent system decreases with increasing substrate concentration (as in the case of simple equilibrium), the situation depicted in Fig. 9 results.

At three concentrations the velocity of formation equals the velocity of breakdown (a, b, and c), so that here the system will be in a steady state. These steady states will be stable in the points a and b only. In these points but not in c small perturbations in the concentrations tend to be damped out (see further ref. 70). Large perturbations in the concentration tend to carry the system from one steady state into another. In combination with the mechanism discussed under (1) a perturbation of sufficient size to exceed the threshold may cause a deviation from concentration that is sufficient to put the system in an other steady state.

When the perturbation is itself a concentration change of some reactant — as in coagulation it is very likely to be — not only the time of exposure to the perturbation will be important, but also its magnitude.

One can imagine how from the site of a lesion — either a wound or an atheromatous plaque — a chemical signal diffuses into the surroundings. At places near the lesion the concentration will be above the threshold that causes a coagulum or a thrombus, and further from the lesion the concentration will drop below that threshold. Thus three general factors influence the size of the coagulum: (a) The power of the source of the procoagulant. This in turn will depend upon the nature of the lesion and the procoagulant potency of the blood. (b) The potency of the anticoagulants. (c) The shape of the gradient of the chemical signal. This is not only influenced by diffusion constants but also by the flow and shape of the vessels (Fig. 10).

Again it must be stressed that this description offers only a general view and does not pretend to be complete. In fact the same mechanism will come

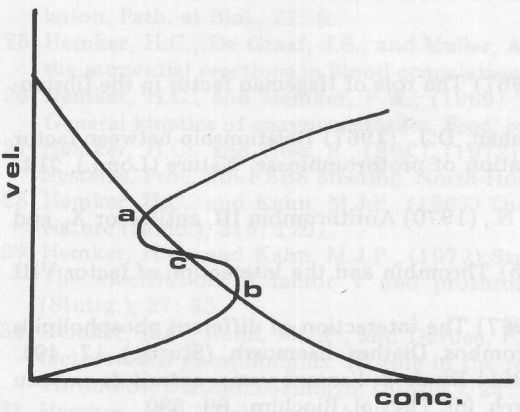

Fig. 9. Multiple steady states in a non-linear system. a and b, stable steady states; c, unstable steady state; conc., concentration; vel., reaction velocity.

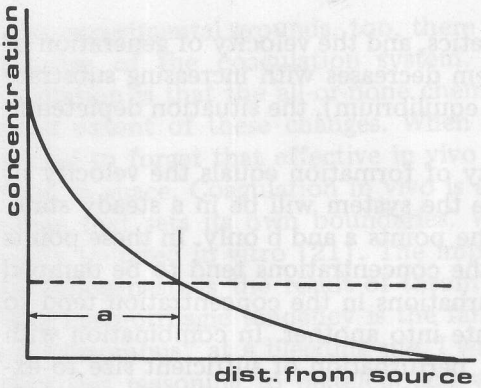

Fig. 10. General factors determining the extent of a limited explosion. Drawn line, gradient of the chemical signal. Dashed line, excitation threshold. a, extent of the "explosion".

into play more often. One can imagine for instance: (a) The enzyme contact product diffusing from a "contact" source and acting upon factor IX while factor IX_a is inactivated by antithrombin 3. (b) ADP diffusing from a localized ATPase in a wound, triggering thrombocytes and being coverted into AMP. (c) Thrombin generated at and diffusing from a thromboplastic site, etc.

As we did in the qualitative part of this chapter, here again we stress only general principles. Clearly the kinetic description of the thrombin-generating system is still in its infancy. We can do no more than indicate a few of the complications that are likely to be encountered in determining the interaction of coagulation factors, and stress the need for an increasingly more detailed description.

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